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Aortic pressure and heart rate in the lobster *Homarus americanus* are modulated by mechanical feedback and neuropeptides

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An Honors Paper for the Department of Biology

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Abstract

Baroreceptors are stretch receptors located in the aorta of mammals; in response to increased afterload, they elicit a decrease in heart rate, creating a negative feedback loop that lowers blood pressure. Although lobsters (*Homarus americanus*) do not have baroreceptors like mammals, closely related land crabs have been shown to have baroreceptor-like responses. Heart contraction is also regulated by the Frank-Starling response, where increasing stretch or preload increases the contractile force of the heart. In addition to these types of biomechanical modulations, lobsters use a central pattern generator, the cardiac ganglion, to maintain synchronicity of the heartbeat. The heart is also controlled by the central nervous system via neuromodulators, such as myosuppressin, which has been shown to increase active force and decrease frequency in isolated lobster hearts. We performed experiments on a lobster heart with the main arteries still intact, and varied the preload by stretching anterior arteries, and the afterload by elevating the dorsal abdominal artery. We added myosuppressin to modulate the cardiac ganglion output and muscle contraction. We found that the baroreceptor-like response is most directly modulated by active force, whereas frequency could be a secondary control. Increasing preload does increase active force, but that does not correlate to a higher cardiac output, which shows that how hard the heart pumps is not what determines how effectively it is pumping. Additionally, we found that myosuppressin has a much stronger effect on frequency than active force, and so with myosuppressin, frequency becomes the main determinant of cardiac output.

Introduction

The cardiovascular system is essential for life across a wide group of organisms. By providing an efficient way to move nutrients to tissues, the heart must closely maintain homeostasis, but also have the flexibility to adapt to an organism's changing environments and needs. One form of that control in mammals is the baroreceptor reflex, which prevents major changes in blood pressure by controlling heartbeat frequency and contractile force of the heart (Klabunde, 2005a). The basis of hearts began developing over 500 million years ago, and has diverged with the many phyla, creating multiple models of a heart in the attempt to do similar jobs. The heart of *Homarus americanus*, the American lobster, is an excellent model system because of its simplicity and similarity to early hearts in the evolution of mammalian hearts (Guadagnoli et al., 2007). Bilaterian animals, one of the last common ancestors between humans and lobsters had hearts like those of modern-day lobsters: less muscular, single chambered, open circulatory system (Stephenson et al., 2017). Understanding the different mechanisms that perform the same tasks can create a better overall understanding of a mammalian cardiovascular system. For example, one way that mammals control cardiac output is through modulation of force and frequency, known as a baroreceptor reflex (Kumada et al., 1990). Even without baroreceptors, other organisms closely related to lobsters have been shown to have baroreceptor-like modulation of their hearts (Burggren et al., 1990). In this paper I investigate control of the lobster heart via extrinsic and intrinsic mechanisms by altering the afterload (extrinsic) and preload (extrinsic), by adding a neuromodulator (intrinsic).

Lobster Cardiovascular Anatomy and Physiology

Unlike mammalian hearts, crustaceans have single chambered hearts that circulate hemolymph, the circulatory fluid, in an open circulatory system in which the heart pumps blood

into exiting arteries, and then blood is pumped through smaller branching arteries, where hemolymph is eventually deposited directly into the tissues (Guadagnoli et al., 2007). The heart pumps oxygenated and deoxygenated blood indiscriminately, relying on the non-specific movement of the blood once it exits the arteries to deliver oxygen/nutrients to tissues.

The lobster heart is suspended by arteries and ligaments within a pericardial space (Maynard, 1960). Hemolymph enters the heart directly from the pericardial space through the ostia, which are valved openings in the wall of the heart itself. These ostia open to draw in hemolymph, and then close during the contraction of the heart. The heart then pumps the hemolymph through seven arteries, which also have valves to prevent backflow: five anterior, one sternal, and one dorsal abdominal artery (DAA) (Maynard, 1960). The lobster heartbeat is regulated by a group of nine neurons that make up the cardiac ganglion, which is a central pattern generator (CPG). These neurons act as pacemaker cells by sending bursts of action potentials to stimulate coordinated contraction of the heart (Dickinson et al., 2015). Pressure and stretch mechanically modulate the heart, which is additionally regulated by the nervous system, via neuromodulators (McMahon and Burnett, 1990). Specifically, we examined control of and by pressure, force and neuromodulation, and their relationship with heartbeat frequency and cardiac output, and how these parameters work together to produce a feedback loop (Figure 1).

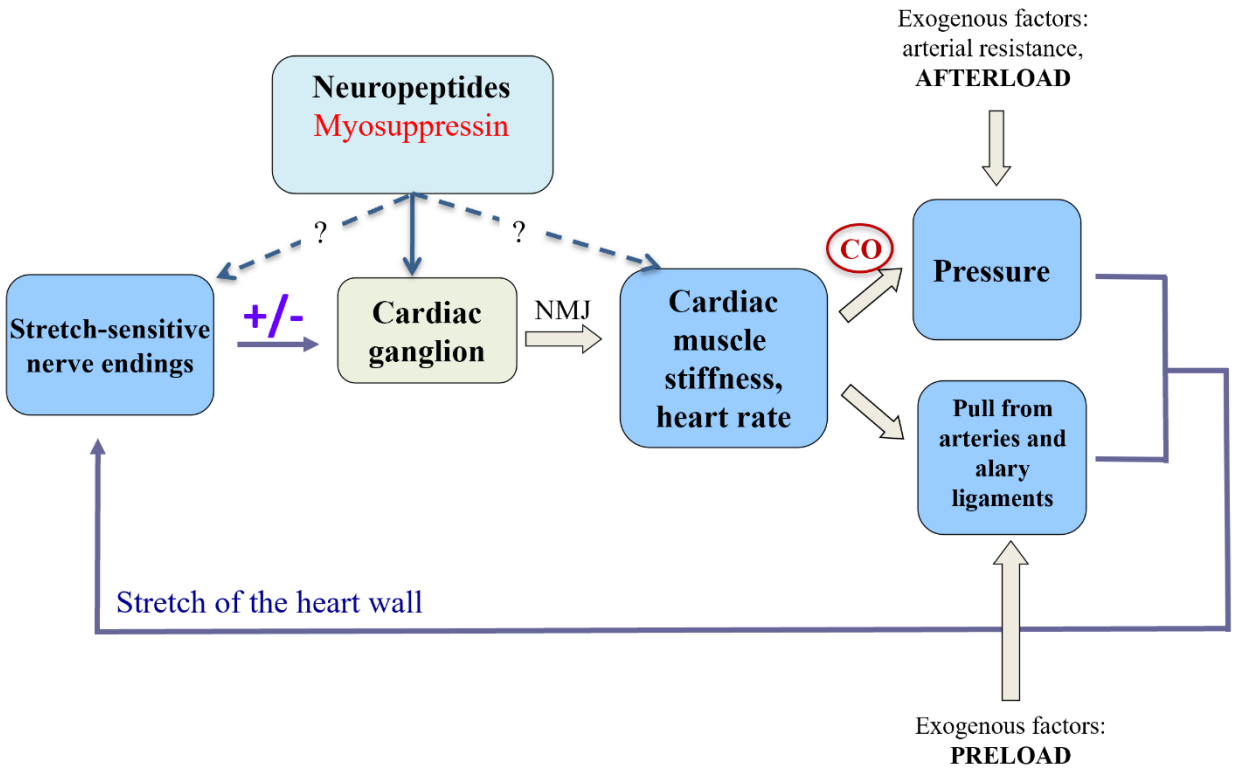

Figure 1. Potential control system of the *H. americanus* heart. The cardiac ganglion, a CPG, sends a signal through the neuromuscular junction (NMJ) to contract at a certain rate, and can alter the stiffness of the muscle, depending on the neuromodulators present (Dickinson et al., 2016). These affect the cardiac output (CO), and therefore the pressure in the heart and the pull on the arteries and surrounding ligaments and consequently stretches the wall of the heart, which activate stretch sensitive nerve endings. The nerves send signals back to the cardiac ganglion to modulate the heart. The central nervous system (CNS) also has some control over the system by releasing neuromodulators, like myosuppressin, which act on both the cardiac ganglion, and on neuromuscular junction or the muscle itself (Stevens et al., 2009). Additionally, external factors such as preload and afterload can also impact the feedback loop.

Central Pattern Generators

The basis of control in this feedback loop (Figure 1) is the cardiac ganglion, a CPG. In general, CPGs are groups of neurons that work intrinsically to produce rhythmic behaviors like walking or swallowing. These signals are sent to motor neurons or muscles through regular bursts of interneurons that make up the CPG (Grillner and Wallen, 1985). They do not need external input from the CNS to generate the rhythms, and the rhythms can be internally modified through feedback loops, and externally modified by altering the strength of a synapse, or a membrane's conductance through chemicals called neuromodulators. The CNS controls the release of the neuromodulators, which creates additional flexibility in the behaviors controlled by

CPGs (Grillner and Wallen, 1985; Katz, 2016). For example, a metronome behaves similarly to a CPG. On its own, the metronome will continue ticking at a constant rate, but you can modulate the speed by changing the distance of the weight from the bottom, or even stop it if you hold it. By changing some properties of the metronome, musicians can regulate the output without controlling every individual tick. In mammalian cardiovascular systems, one way of modulating the "CPG" in the heart is through the baroreceptor reflex.

Baroreceptor Reflex

Baroreceptor cells are mechanoreceptors located in the mammalian aortic arch and carotid sinus, which are short-term modulators of pressure in the cardiovascular system. Essentially, the baroreceptor reflex acts as a negative feedback loop by signaling the CNS to activate different processes in an effort to reduce fluctuations in pressure (Dampney, 2017; Kumada et al., 1990). For example, if the blood pressure is too high, the baroreceptor will send a signal to the CNS, which will then cause a cascade release of hormones and peptides that cause a decrease in peripheral artery resistance, frequency, and stroke volume (volume of blood ejected in a single heartbeat), which leads to a decrease in the arterial pressure (Kumada et al., 1990). The baroreflex response does not regulate blood pressure to one specific range but has dynamic flexibility in the range it regulates to because it would be disadvantageous to always regulate blood pressure to one specific range. To effectively adapt to a dynamic environment, it was evolutionarily advantageous to have a baroreflex that could effectively regulate blood pressure for a range of situations. For example, when someone is sleeping they don't need to replenish their cells' nutrients as quickly as someone who is running (Dampney, 2017).

Crustaceans lack the specific baroreceptor cells that are found in mammals, but they seem to have similar mechanoreceptors that modulate changes in hemolymph pressure (such as in

Figure 1). Land crabs, *Cardisoma guanhumi*, have been seen to have a baroreceptor-like response due to a change in hemolymph volume (Burggren et al., 1990). These baroreflex-like responses can occur within several heartbeats, which could imply that the pathway is controlled with neuromodulators (McMahon and Burnett, 1990). Similar results were found in *Carcinus maenas*, where decreasing hemolymph levels, and therefore pressure, caused contraction of the dorsoventral muscle, and infusion of saline increased muscle relaxation, a response that mimics that of a baroreflex (Taylor and Taylor, 1991). Lobsters have cardiovascular systems similar to those of these crustaceans, so lobsters could possess a similar feedback system.

Afterload (pressure) and Preload (stretch)

The pressure that causes the mammalian baroreceptor response is the afterload. This parameter is the pressure that the heart pumps against to push blood out of the heart and into surrounding arteries, and it is not exclusive to mammals with baroreceptors. A change in afterload impacts the end systolic volume, or the residual blood left in the heart. This, in turn changes the stroke volume, or the blood pumped out of the heart, and therefore the cardiac output (the rate of blood pumping):

$$\text{Cardiac Output} = \text{Stroke Volume} \times \text{Heart Rate}$$

In lobsters, it has been shown that the afterload is modulated through the stretch receptors that are found in the cardiac ganglion, which therefore also have control of the cardiac output (Dickinson et al., 2015) (Figure 1). Other crustaceans have shown similar control mechanisms, such as the *Ligia pallasii* which found that their cardiac ganglion neurons are sensitive to changes in stretch within the heart (Sakurai and Wilkens, 2003).

The end diastolic volume is the volume in the heart when it is filled with blood, right before contraction, i.e., when the heart is at its largest volume. The ability of the heart to stretch therefore directly impacts the end diastolic volume. The stretch experienced by the walls of the heart is also called the preload, and it can be manipulated by external stretch of ligaments (Rose et al., 2001). There are two mechanisms by which this portion of the system is modulated: cardiac ganglion via stretch receptors, and Frank-Starling Response. The cardiac ganglion modulates the preload by changing muscle stiffness and/or heartbeat frequency. Since the end diastolic volume is the maximum blood in the heart at one time, it also directly impacts stroke volume due to the Frank-Starling effect.

Frank-Starling Effect

Frank-Starling mechanism refers to the effect whereby change in muscle unit (i.e., sarcomere) length changes the force with which the sarcomere can contract. A sarcomere contracts due to the overlap of actin and myosin proteins, which bind together and shorten the muscle fiber. The overlap between the actin and myosin therefore determines the force with which a muscle can contract (Sweeney and Hammers, 2018). If the sarcomere is the right length, there is maximum overlap, which generates a maximum force. On the scale of a heart, the force is related to the stroke volume, and the length is related to pressure in the heart chamber, which means that increasing the preload causes an increase in the stroke volume (Klabunde, 2005b). Regulation of the heart is centered around the rate at which blood is moving through the system or the cardiac output.

Cardiac Output

The purpose of the cardiovascular system is to circulate nutrients and oxygen by moving blood or hemolymph. Cardiac output is the variable that is determining the distribution of

nutrients, it is the parameter that all these mechanisms are attempting to regulate. As previously stated, the cardiac output is directly related to the stroke volume and frequency. The stroke volume itself is modulated by preload (and therefore force) and afterload (and therefore pressure) due to two different mechanisms. Because increasing preload is effectively lengthening the muscle, so through the Frank-Starling Law dictates that higher preloads cause increase of end diastolic volume, and therefore stroke volume. Increasing afterload modulates the pressure-volume curve in the heart. Increased afterload means that the pressure in the aorta is higher, which means that the heart must isovolumetrically contract for longer so the blood pressure in the heart is higher than that outside the heart. Then when the heart ceases contraction, the valve between the heart and the aorta closes due to the pressure in the heart falling below that of the aorta. Increasing the afterload overall decreases the amount of time that the heart is able to pump blood out of the heart, and therefore the stroke volume is decreased at increased afterloads. In addition to biomechanical regulation, lobsters can regulate their hearts via the nervous system by the release of neuromodulators.

Neuromodulators and the Effects of Myosuppressin

Neuromodulators are a large class of molecules that are released by neurons and interact with neurons and non-neuronal cells, depending on the particular modulator. Additionally, the time frame of effectiveness varies between specific neuromodulators. For example, GABA is a short-term inhibitor in the brain in mammals, whereas gonadal steroids hormones cause sexual dimorphism throughout the body when there is long term exposure (Borde et al., 2020). The wide range of these molecules allows there to be specific control over CPGs, which allows for more effective adaptation to different environmental conditions (Dickinson, 2006).

pQDLDHVFLRFamide, or crustacean myosuppressin, is a peptide hormone that has been shown to cause an increase in force and a decrease in frequency of isolated hearts in *H. americanus.* The effect of myosuppressin is dose dependent, with statistically significant changes to the frequency and the force at 10^{-7} M, which is similar to the *in vivo* concentration. It was determined that myosuppressin has direct effects on the cardiac ganglion, and also has effects on the neuromuscular junction and surrounding tissues (Stevens et al., 2009). Typically, force and frequency are loosely related, both increasing or decreasing at different stimuli, but myosuppressin could be incredibly useful to dig deeper into the control mechanism of the heart because it allows the effects of frequency and force to be examined separately.

Hypotheses

Using *H. americanus* as a model system, I explored the mechanistic relationship of afterload, and/or preload on the cardiac output, frequency, blood pressure, and force to ask the question: Does the lobster heart exhibit a baroreceptor-like response. The relationship between these variables was also explored by de-coupling frequency and force, two variables which are typically correlated in the baroreceptor response, via the addition of the neuromodulator myosuppressin. My hypotheses are: (1) increasing afterload will increase lobster blood pressure, (2) if there is a baroreceptor response, the heart system will respond to increased blood pressure by decreasing contraction force and contraction frequency, which should buffer the cardiac output and blood pressure response to create a flexible yet controlled system; (3) increasing preload will increase stretch on heart and therefore create a Frank-Starling effect, which increases contraction force and pressure; and (4) addition of myosuppressin, which decreases frequency and increases force, will cause a decreased baroreceptor response because the frequency would not increase as it typically does when a baroreceptor response is induced.

Materials and Methods

Source and Maintenance of Lobsters

Lobsters, *Homarus americanus*, were obtained from a local seafood retailer in Brunswick, Maine. Overall, there were 36 lobsters (17 male and 19 female), with an average carapace length of 11.1 cm (Table 1). They were maintained in circulating natural seawater kept between 10-12 °C and had 12-hour light/dark cycles before dissection. The lobsters were fed on a weekly basis either shrimp or scallops. The lobsters were numbered according to dissection date, with the first 10 from another previous student's honors paper (data not included in paper). The lobsters were numbered by date. Since the three preparations were not done individually, the numbers for each individual experiment are not sequential.

Table 1. Distribution of lobsters in each experiment, male to female ratio, and the average carapace length for each subset of experiments. Afterload manipulation experiments are completed at a low and high afterload. Preload and afterload manipulation experiments are completed at six preloads, with a low and high afterload at each preload. Myosuppressin afterload are experiments completed at a low and high afterload with and without myosuppressin.

Experiment	Number of Lobster (male:female ratio)	Average carapace length (cm)
Afterload Manipulation	18 (8:10)	11.2
Preload and Afterload Manipulation	17 (8:9)	11.3
Myosuppressin Afterload	18 (5:13)	11.6
Overall:	**36 (13:23)**	**11.4**

General Dissection

H. americanus were anesthetized on ice for 45-60 minutes before dissection. Sex was determined and carapace length was measured (±0.5 cm). The anterior cephalothorax, claws, legs, and swimmerets were removed before the lobster was pinned ventral side up into a bath of lobster saline (composition mmol 1^{-1}: 479.12 NaCl, 12.74 KCl, 13.67 $CaCl_2$, 20.00 $MgSO_4$, 3.91 Na_2SO_4, 11.45 Trizma base, and 4.82 maleic acid; pH: 7.45, (Dickinson, 2014)) in a 9x12 tray with a sylgard bottom. The tray was maintained between 11 and 14 °C, via aluminum water

cooling blocks, which were connected to a Fischer Scientific Isotemp Chiller. Once the lobster was secured in the saline bath, the tail muscle, digestive tract, and reproductive organs were removed to expose the intact beating heart and arteries of the lobster.

Experimental Set-Up

The goal of this preparation was to have flow go into the ostia and exit out of the DAA through a tube that could be used to alter the afterload, to measure the heart volume flow rate, and to measure pressure. The five anterior arteries were tied together with suture silk (weight 0-6). Flow exiting the heart was restricted to the DAA by further tying off the sternal artery and the lateral arteries branching off the DAA (Figure 1B & 1C).

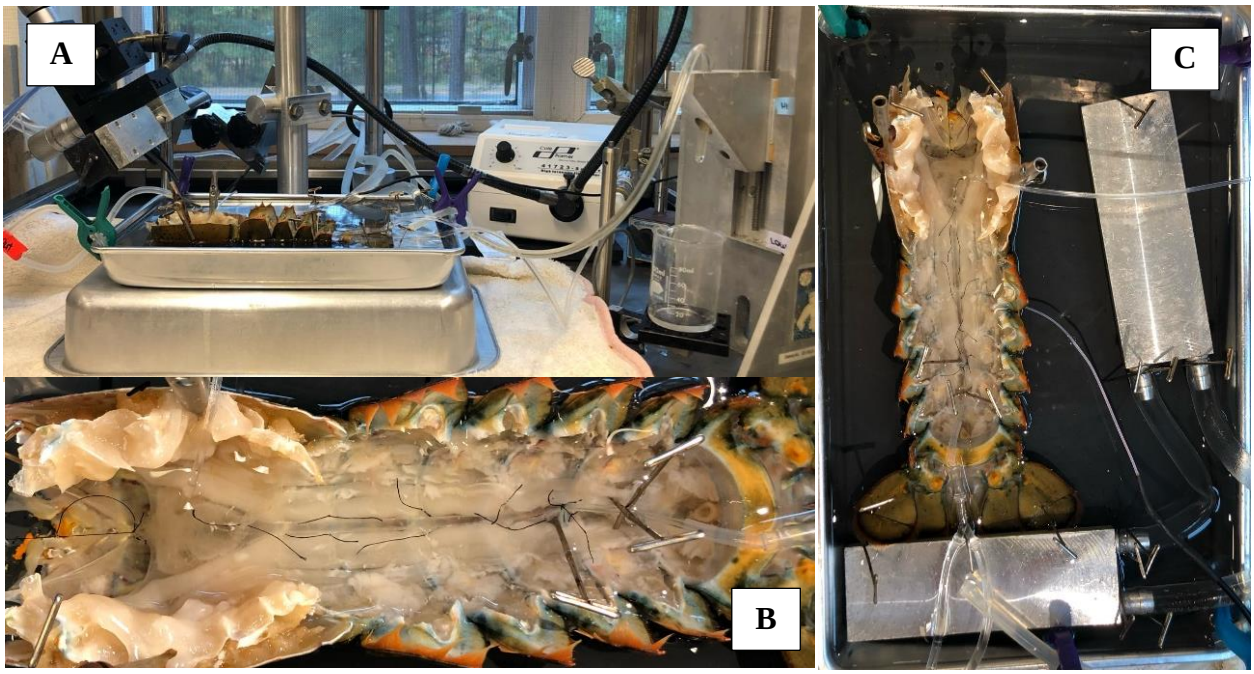

Figure 2. Overall view of set up (A), with a lateral view of the dissected lobster. On the left the grey and black box is a force transducer, and on the right is the grey Velmex unislide stage, which is used modulate afterload, and below it rests a force transducer and beaker to measure cardiac output. (B) A ventral close-up of the dissection. On the left the five anterior arteries are tied off with suture silk that connects to the force transducer. The top ostia is canulated with a tube infusing saline. The DAA exits on the posterior end of the heart, and travels along the tail, with six sets of branching arteries. Those arteries are tied off with suture silk and the end of the DAA is canulated with a small tube that measures pressure and flow rate. (C) A ventral view of entire dissection tray. The grey blocks maintain temperature of the saline, and posterior to the lobster the tube branches with one end measuring cardiac output and the other pressure.

The five anterior arteries were connected to the lever of an Aurora Scientific force transducer (Dual-Mode Lever Systems, Model 300C, Aurora Scientific, Aurora, ON, Canada) via suture silk, which was used to measure the force of contraction and to increase the longitudinal pull, or preload, on the heart. The end of the DAA was cannulated to measure pressure and flow rate, and to change the afterload by changing the elevation of the end of the cannula using a Velmex unislide stage. The cannula split via a Y-connector, sending one end to the pressure transducer, and the other end through the Velmex unislide stage before flowing into the 100 ml beaker on a pressure plate (Omega Engineering LCAE 600G Single Point Load Cell). Pressure was measured using an Omega Pressure Transducer (Stamford CT 06907). The signals for the length (preload), force, and pressure were first filtered (with Model 44 Brownlee Precision Instrumentation amplifier, with lowpass filters of 10 kHz, 10 kHz, and 20 Hz respectively), then recorded (by Cambridge Electronic Design (CED) Micro 1401), and saved/viewed with Spike2, version 9.0.2.0 software (CED, Cambridge, UK). The stroke volume was filtered with an OMEGA DMD-519 high performance strain gage amplifier, and then recorded (CED Micro 1401), and saved/viewed with Spike2. Cardiac output was measured by collecting the stroke volume of the heart into the beaker, recording the change in weight over time, which was used to derive cardiac output (where 1 g saline = 1 mL of saline).

A perfusion system (Masterflex L/S Compact Drive Model 77240-30, 200 RPM) maintained a constant level of fresh lobster saline in the experimental tray. For experiments with myosuppressin, one of the exposed ostia was cannulated and perfused with lobster saline or lobster saline with 10^{-7} M myosuppressin with a flow rate of 6.4 mL/min (Gilson Minipuls 2).

Variation in Afterload and Preload

To vary afterload, the height of the end of the DAA cannula was alternated between the height of the lobster heart (low), and the height at which the heart was able to cause saline to drip into the beaker every 3 or 4 heartbeats (high), with a maximum height of 12 cm. Each afterload pressure was maintained for about 200 s; each set of high and low (high-low) afterloads was replicated five times for an afterload experiment. For the preload experiments, preload was altered by extending the length of the heart by 0.5 mm at a time, up to 2.5 mm (for a total of 6 preloads). One set of high-low afterloads was completed for each preload.

Addition of Neuromodulators

The neuromodulation experiment was completed using the same preparation as the preload/afterload preparation with an additional cannula in the ostia. First three sets of high-low afterload were recorded, while saline was injected through the ostia's cannula. Then the saline was switched to lobster saline containing 10^{-7} M myosuppressin; the tray of saline was also exchanged for saline with myosuppressin of the same concentration. Myosuppressin was perfused for 15 minutes before recording to ensure maximum effects, and then another three sets of high-low afterloads were recorded. Next the myosuppressin was washed out for an hour, before a control and myosuppressin condition with two more sets of high-low afterload each were recorded, with the same process of application of myosuppressin (Stevens et al., 2009) (Figure 3).

Figure 3. A diagram of the timeline of the myosuppressin experiment. First 3 control high-low sets, then the myosuppressin is added for 15 minutes. A set of 3 high-low afterloads were recorded for the myosuppressin treatment. Then the myosuppressin was washed out for an hour. A second control trial was completed with 2 sets of high-low afterloads, and finally myosuppressin was perfused for 15 minutes before a second myosuppressin trial was completed with two sets of high-low afterloads.

Data Analysis

Inclusion/Exclusion Criteria: Myosuppressin Data

Datasets (n=4) were excluded if the hearts had bad rhythms, such as slow, (period >1.5 s during initial control trial) weak heart beats or double beating. Weak hearts are characterized by how the muscle contracts during each heartbeat, a weak heartbeat had minimal movement during contraction. They were also excluded if the heart was not able to have a difference in the maximum pressure at low afterloads and minimum pressure at high afterloads on the second myosuppressin trial, where the heart would be the weakest, at the end of the experiment. With these inclusion/exclusion criteria, the sample size was reduced to n=18.

Afterload Trials: Frequency Analysis

Using *Mathematica* 11.3 (Wolfram Research, Champaign, IL, USA) programs written by O. Ellers, each peak of the force time graph was identified. The time between each peak was determined and averaged for each of the 10 different trials, with five at high afterload and five at low afterload on each lobster (Figure 4). The high and low afterloads across the five trials were then each averaged for each lobster. These data were exported into Graphpad Prism Version 8, where a 2-way ANOVA was run to determine if the frequency changed between the high and low afterload pressures overall, and for each lobster.

Afterload Trials: Pressure, Force, and Cardiac Output

An additional program was written by O. Ellers to determine the cardiac output by integrating the flow rate extracted in Spike2 (Figure 2A). In the same program, the diastolic (minimum), systolic (maximum), and the pulse (amplitude) pressures were averaged over the length of each trial. In a separate program, the systolic, diastolic, and active of the forces exerted by the heart were averaged for each afterload and trial (Figure 2B and 2C). These data were exported into GraphPad Prism Version 8, where a 2-way ANOVA was performed to determine if there was a significant difference in high and low afterloads within each lobster for each of the pressures, forces, and the cardiac output. Percent change from low to high afterloads was manually calculated. If the t-test was statistically significant, then the mean percent change was calculated by GraphPad Prism. Percent change outliers were identified (ROUT Q=1%) and excluded from statistical analysis. With the cleaned data, outliers were checked again to ensure that all outliers were caught.

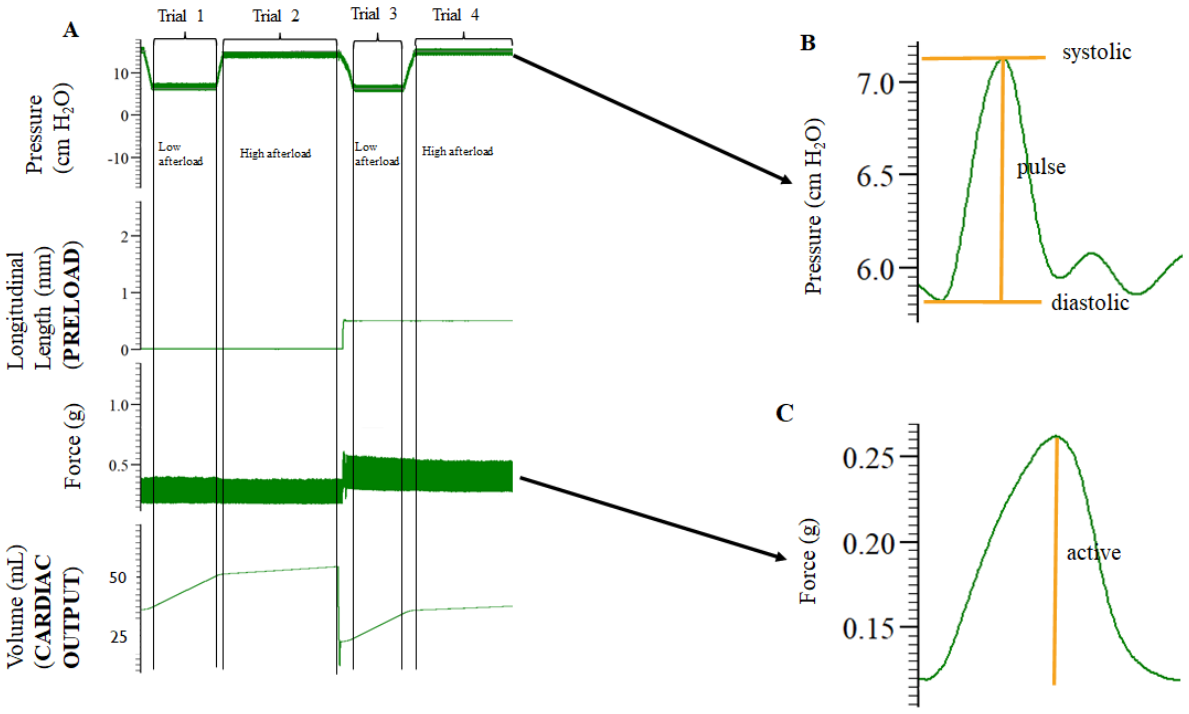

Figure 4. **Annotated Spike2 recording of preload experiment at two preload lengths.** An overview of all the graphs (A), with pressure, longitudinal length (preload), force, and volume (cardiac output). A high-low afterload pair constitutes a trial. Pressure (B) and force (C) graphs were enlarged to one heartbeat to define period, active force, pulse pressure, and systolic and diastolic pressure/force.

Preload Trials: Pressure, Force, Frequency, and Cardiac Output

The data from the preload trials were first analyzed as the afterload experiments were, calculating the average cardiac output, frequency, the diastolic, systolic, and pulse pressure, diastolic, systolic, and active forces at each preload for each lobster. In a separate program, the data for a particular parameter were imported, and to enable comparisons across lobsters, data were normalized to the maximum value for each parameter within a given lobster. The relative values were then averaged over all the lobsters for each parameter, and the standard error of the mean was calculated. These data were exported to GraphPad Prism and analyzed with a 2-way ANOVA to determine if there were any significant differences between the high and low afterloads at the various preload stretches. Additionally, a Spearman Rank test was used at each afterload to determine the significance of preload on each the parameter. The test was performed

in a Mathematica program written by O. Ellers. Frequency was additionally analyzed for each lobster, and a simple linear regression was performed on each lobster, and the lobsters were split into three categories: no change, negative, and positive.

Myosuppressin Trials: Pressure, Force, Frequency, and Cardiac Output

The data from the myosuppressin trials were analyzed using similar programs and techniques as the preload trials, by first finding averages for each parameter for each condition (i.e., control 1, or myosuppressin 1), and then normalizing the data within each lobster in Mathematica programs. The data were exported to GraphPad Prism, and 3-way ANOVA and post hoc tests were used to determine significant differences between the four conditions (first control, first myosuppressin, second control, second myosuppressin) at high and low afterloads.

For each parameter, the Mathematica program would look at a certain range, called nn, of values before and after a set point to calculate the value of a specific parameter for a single heartbeat. For example, to find the systolic pressure, the program would look at 60 points, or 0.6 seconds (due to the sampling rate) before and after a chosen point to find the maximum pressure value in that section. Then the maximum pressures were averaged for a particular trial. With preload and afterload only manipulation, the period does not change too much, so the value was set at 60 (or a period of 1.2 s), but with the addition of myosuppressin, the variability of the frequency increased, so to optimize the length of nn without manually changing it for each trial, the following formula was used:

$$nn = \frac{mean\ period + (2\ \times standard\ deviation)}{2} \times 100$$

Results

Afterload Experiments

As expected, the cardiac output of all 19 lobsters decreased with increased afterload ($p<0.0001$, paired t-test, Figure 5A). The percent change decrease was between 44.63% and 93.45%, with an average decrease of 76.94±2.8% (Figure 5B).

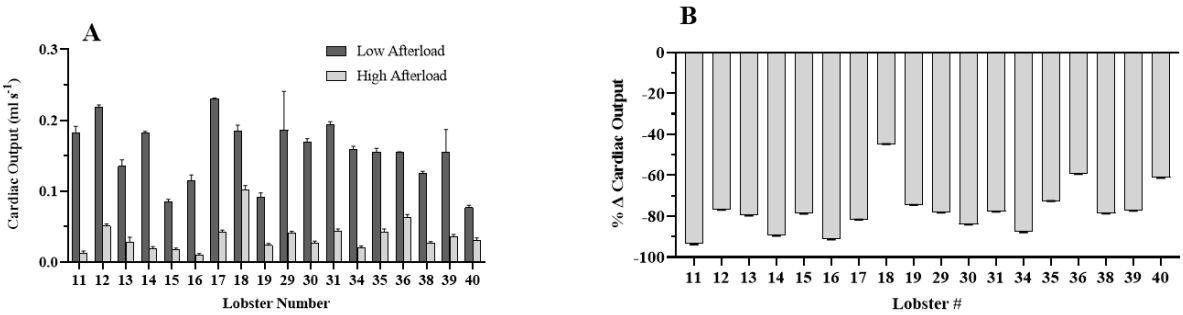

Figure 5. Cardiac output was significantly decreased by increasing the afterload ($p<0.0001$, paired t test). (A) the cardiac output at two afterloads, and (B) the percent change for each lobster. The percent change from low to high afterload was decreasing between 44% and 93% for all lobsters. Error bars represent one standard error.

Overall, there was no significant change in frequency, but the response to pressure and stretch was variable, but in nine of the 18 lobsters had a small insignificant positive change ($p=0.36$ paired t-test, Figure 6A). There were three outliers with significant differences, lobster 18 and 29 which increased frequency and lobster 39 which decreased frequency (Figure 6B).

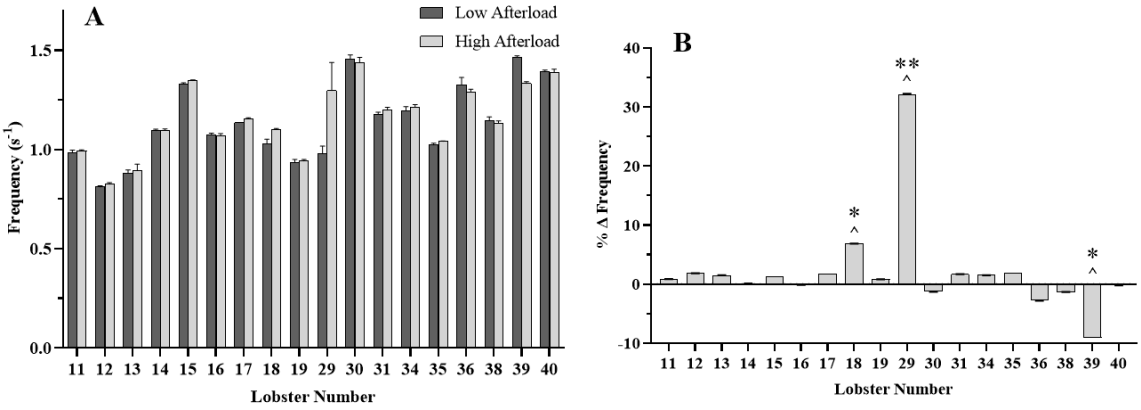

Figure 6. Frequency of heart was not significantly affected by afterload changes, except for three outliers (p=0.36 paired t-test). (A) The frequency at high and low afterloads of each lobster, and (B) the percent change for each lobster. Error bars represent one standard error, ^ indicates an outlier (ROUT Q=1%), and * indicates $p < 0.05$, and ** indicates $p < 0.0001$ (2-way ANOVA, post hoc).

The high afterload increased the systolic and diastolic pressures of the heart (Figure 7A, $p<0.0001$ & 6B, $p<0.0001$, paired t-tests), with an average increase of 193.6±22.5% for diastolic pressure (Figure 7D) and 134.6±10.9% for systolic pressure (Figure 7E). The pulse pressure did not change consistently with afterload modulation (Figure 7C, $p=0.68$, paired t-test), with four lobsters insignificantly decreased pulse pressure at higher afterloads, and nine lobsters insignificantly increased pulse pressure at afterloads (Figure 7F). Five lobsters had significantly changed in pulse pressure with increasing afterload, with two pulse pressures increasing, and three decreasing (Figure 7F).

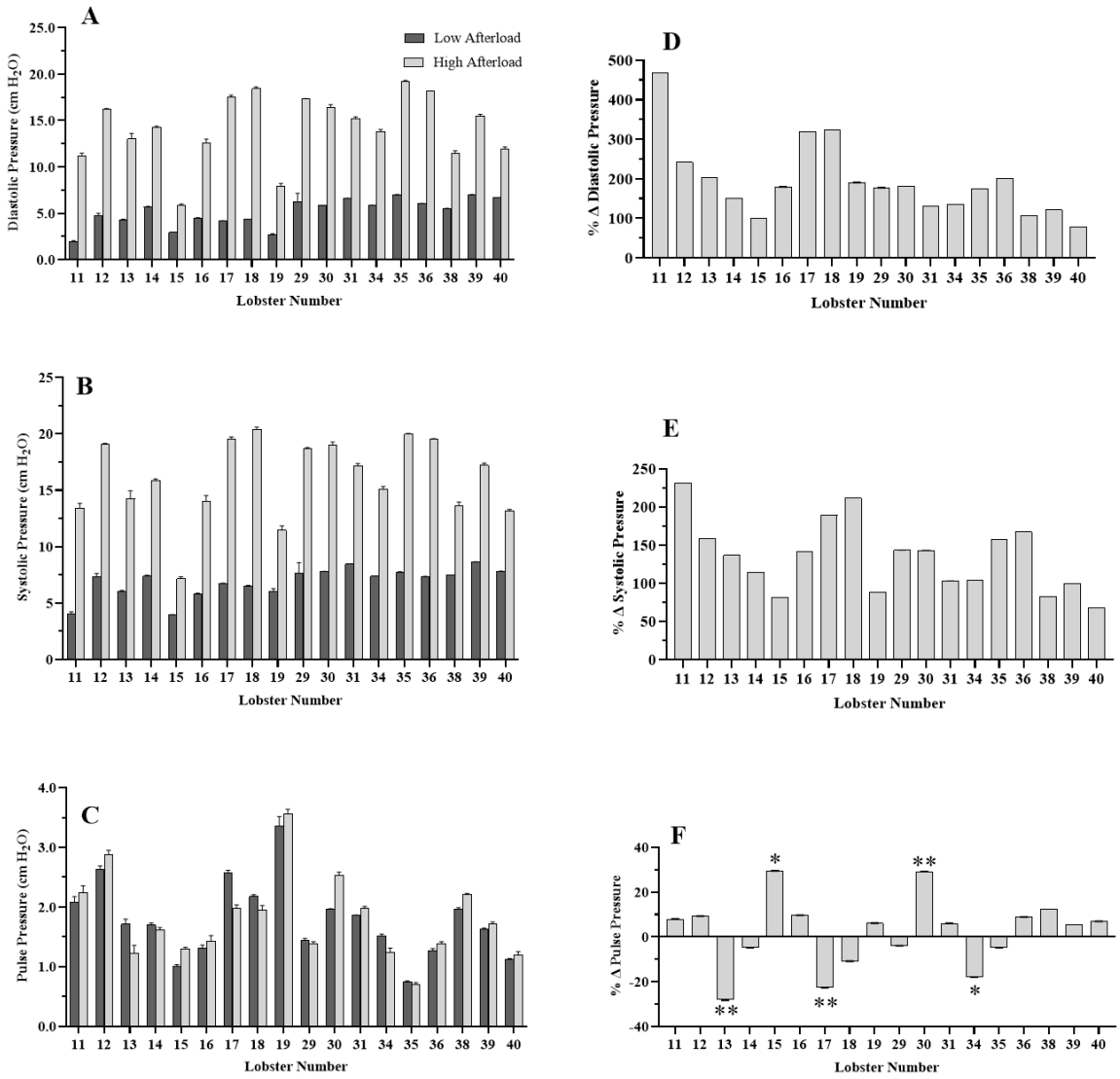

Figure 7. The pressure of the heart increased with increased afterload pressure, for diastolic (A), systolic (B), but not pulse (C) pressures. Additionally, the percent changes for the diastolic (D), systolic (E), and pulse (F) pressures for each lobster are shown. Increasing afterload significantly increased diastolic ($p<0.0001$, paired t-test) and systolic pressures ($p<0.0001$, paired t-test). Pulse pressure did not consistently change across lobsters with increased afterload ($p=0.64$, paired t-test), but individually, three lobsters had a statistically significant decrease in pulse pressure and two had a statistically significant increase in pulse pressure. Error bars represent one standard error, and * indicates $p<0.05$, and ** indicates $p<0.0001$ (2-way ANOVA, post hoc).

Increasing the afterload caused a statistically significant decrease in diastolic (Figure 8A, $p=0.040$, paired t-test), systolic (Figure 8B, $p=0.0054$, paired t-test), and active force (Figure 8C, $p=0.0015$, paired t-test). With increasing afterload, the diastolic force decreased an average of

2.7±0.7% (Figure 8D), systolic forces decreased an average of 4.7±0.9% (Figure 8E), and active forces decreased an average of 12.7±3.1% (Figure 8F).

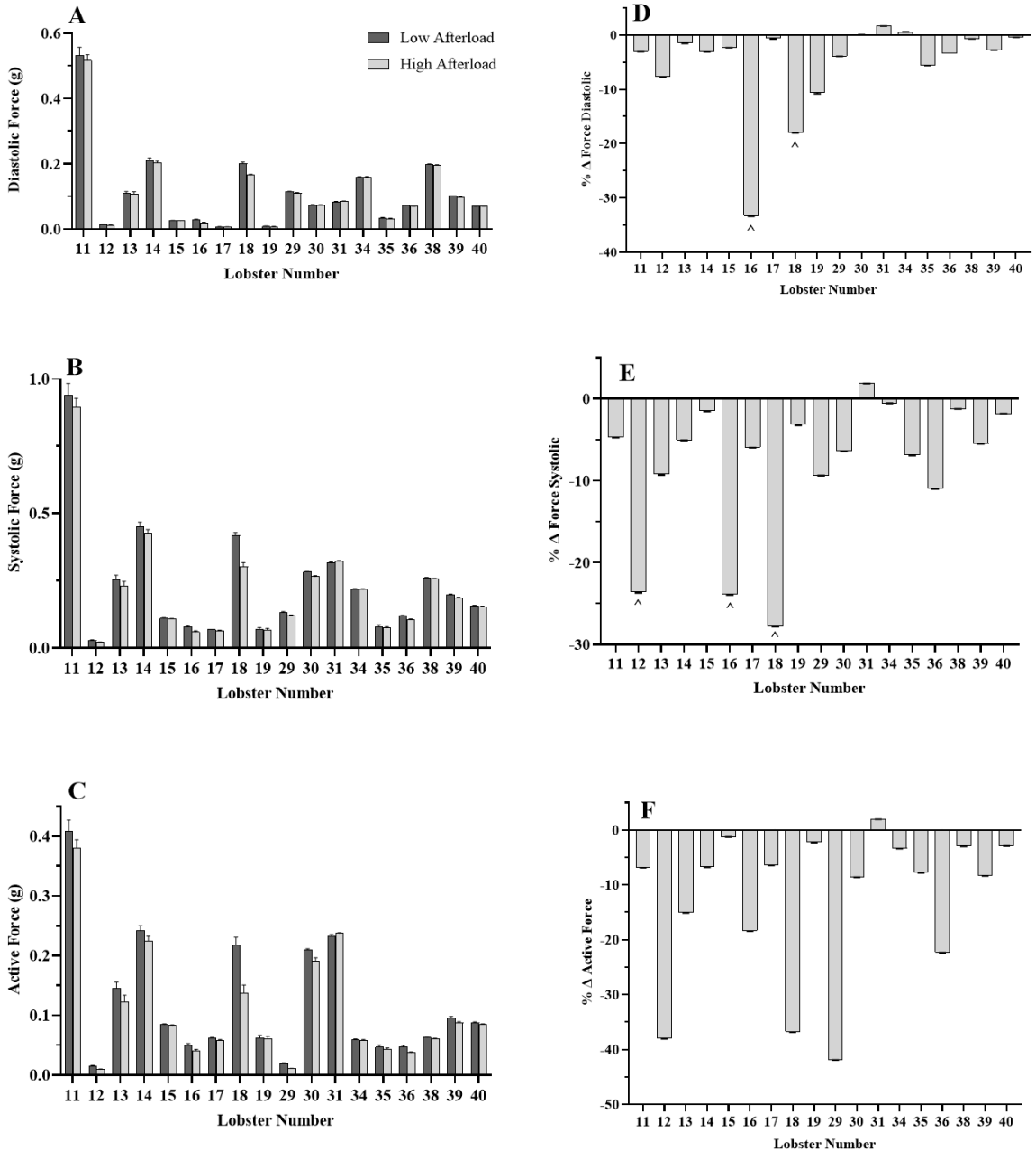

Figure 8. Increasing afterload generally decreased in diastolic (A), systolic (B), and active (C) force. Diastolic force increased in two lobsters, but the increase was less than 2% both times, and decreased a maximum of 33% with outliers, or 10% without them (D). The systolic force decreased at most 30% with outliers, or 11% excluding them, and increased no more than 2% (E), and the active force decreased by no more than 41% and increased no more than 2% (F). Overall, the diastolic (p=0.040, paired t-test), systolic (p=0.0054, paired t-test), and active (p=0.015, paired t-test) significantly decreased. Error bars represent one standard error, and ^ indicates an outlier (ROUT Q=1%).

Preload Experiments

Increasing preload also reduced cardiac output at low afterloads ($p=3.8 \times 10^{-10}$, Spearman Rank), but not at high afterloads (p=0.23, Spearman Rank). Overall, increasing the afterload decreased the cardiac output (Figure 9, p<0.0001, 2-way ANOVA).

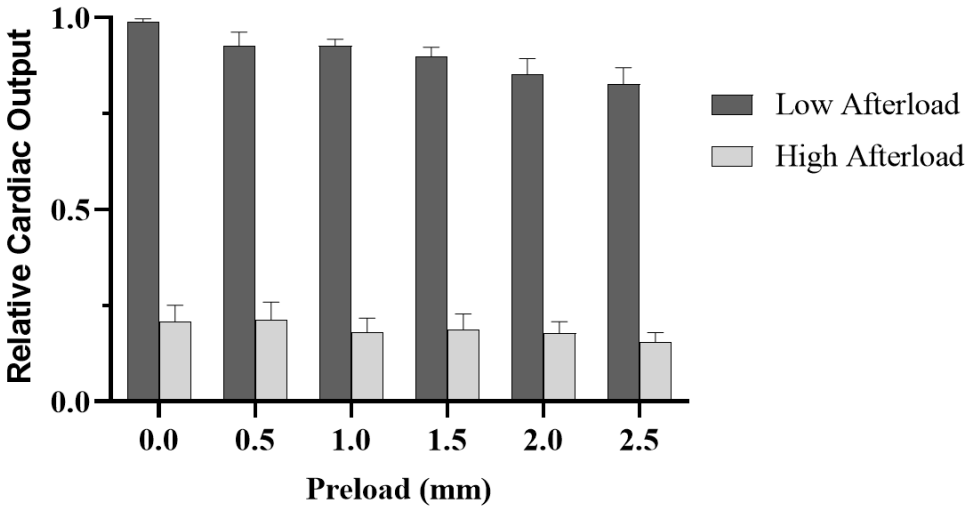

Figure 9. Increasing preload decreased the relative cardiac output at low afterloads only, but not high afterloads, and increasing the afterload decreased the relative cardiac output at every preload. Overall, the increase in afterload caused a decrease in cardiac output (p<0.0001, 2-way ANOVA). At a low afterload (dark grey bars), the increase in preload decreased the relative cardiac output ($p=3.8 \times 10^{-10}$, Spearman Rank), but not at high afterloads (light grey bars, p=0.23, Spearman Rank). Error bars represent one standard error.

The effects of preload on frequency were highly variable among lobsters. Because of this, the overall average did not display the different response types of the frequency. In 53% of lobsters, frequency did not change in response to change in preload at either preload (Figure 10A), in 18% of lobsters, frequency increased due to the change in preload at both afterloads (Figure 10B), and in 18% of lobsters, frequency decreased due to a change in preload at both afterloads (Figure 10C).

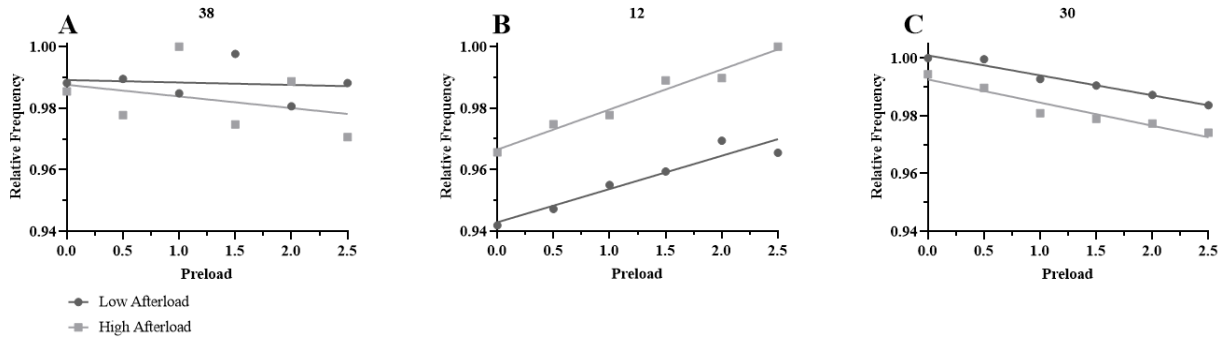

Figure 10. Examples of frequency with no change (A), increase (B), and decrease (C) with increasing preload. A simple linear regression was used to determine if the slope was significantly different from zero. Overall, nine lobsters had no significant change in frequency due to increased preload, three had increased frequency due to increased preload, and three lobsters had decreased frequency due to increased preload. Two lobsters had inconsistent changes in frequency across afterloads (one afterload had an increase in frequency and the other had no change). Graphs are labelled with the lobster number the data are from.

The frequency was not significantly affected by afterload changes overall (p=0.31, 2-way ANOVA) or at specific preloads (post hoc analysis). Preload also did not elicit changes in contraction frequency (Figure 11, p=0.56 high afterload, p=0.61 low afterload Spearman Rank).

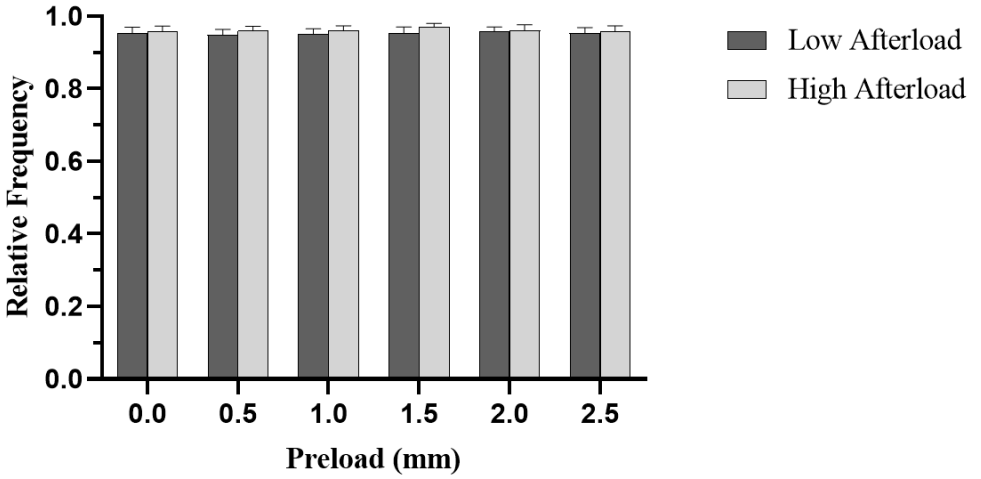

Figure 11. Relative frequency at five different preloads (0 to 2.5 mm at 0.5 mm increments). Relative frequency had no significant difference between low and high afterloads for any preload overall (p=0.31, 2-way ANOVA). At high and low afterloads, changing the preload had no effect on the frequency of the heartbeat (p=0.56 high afterload, p=0.61 low afterload Spearman Rank). Overall, the frequency was not significantly affected by changes in pressure or stretch on the heart. Error bars represent one standard error.

Both diastolic and systolic pressures significantly increased at higher afterloads overall (Figure 12A & 12B, p<0.0001, 2-way ANOVA for both diastolic and systolic). However, diastolic, and systolic pressure decreased as preload increased at high afterloads, but not at low

afterloads (diastolic high: p=0.0028, systolic high: p=9.0 x 10^{-6}, diastolic low: p=0.95, systolic low: p=0.61, Spearman Rank). The pulse pressure was not significantly affected by the afterload (Figure 10C, p=0.67, 2-way ANOVA), but increasing preload significantly decreased pulse pressure at both low (p=4.0 x 10^{-6}, Spearman Rank) and high afterloads (p=1.5 x 10^{-4}, Spearman Rank).

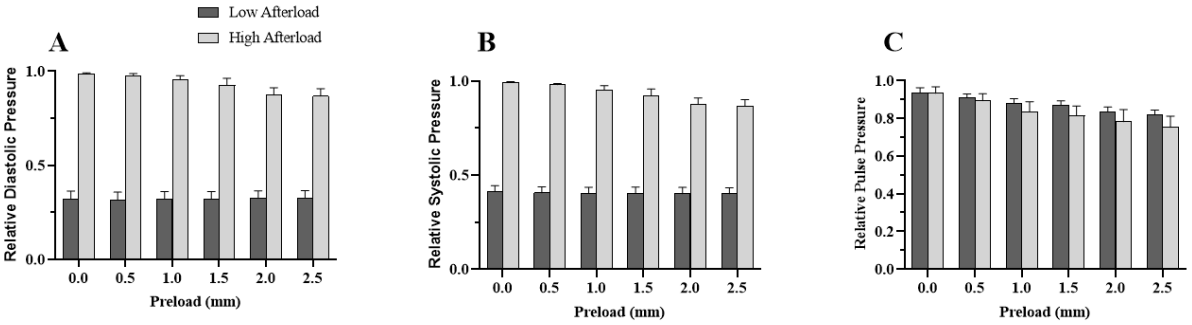

Figure 12. Effect of increasing preload on diastolic (A), systolic (B), and pulse (C) pressures at two afterloads. Increasing afterload significantly increased diastolic (p<0.0001, 2-way ANOVA), and systolic pressures (p<0.0001, 2-way ANOVA), and has no effect on pulse pressures (p=0.67, 2-way ANOVA). At high afterload pressures, changing the preload changed the diastolic (p=0.0028, Spearman Rank), systolic (p=9.0 x 10^{-6}, Spearman Rank), and pulse pressures (p=1.5 x 10^{-4}, Spearman Rank). At low afterloads, changing the preload did not cause change in the diastolic (p=0.95, Spearman Rank), or systolic pressures (p=0.61, Spearman Rank), but did effect low afterload pulse pressure (p =4.0 x 10^{-6}, Spearman Rank). Error bars represent one standard error.

Increasing afterload significantly decreased relative diastolic (Figure 13A, p=0.0002, 2-way ANOVA), systolic (Figure 13B, p=0.0002, 2-way ANOVA), and active (Figure 13C, p=0.014, 2-way ANOVA) force across all preloads. At low and high afterloads, increasing preload elicited an increase in the diastolic force (low: p=9.2 x 10^{-43}, high: p=9.0 x 10^{-39}, Spearman Rank), the systolic force (low: p=8.8 x 10^{-35}, high: p=9.0 x 10^{-38}, Spearman Rank), and active force (low: p=8.8 x 10^{-34}, high: p=8.2 x 10^{-26}, Spearman Rank). Additionally, the effect of preload on the three parameters (diastolic, systolic, and active forces) were similar at both high and low afterloads (simple linear regression p=0.24, p=0.38, and p=0.43 respectively).

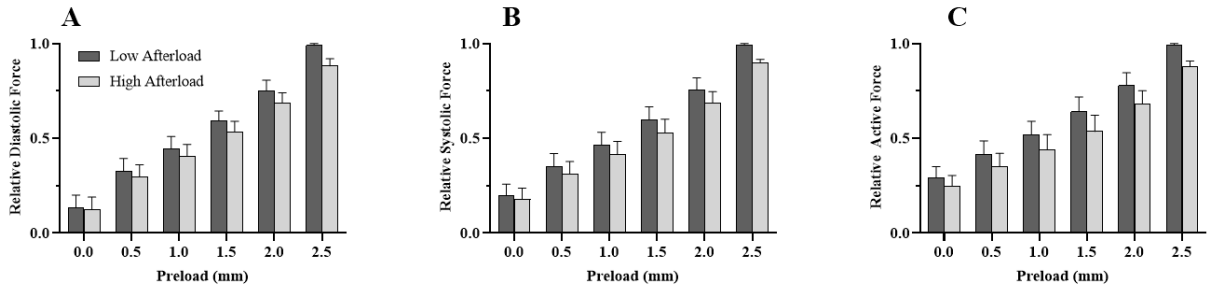

Figure 13. The effect of preload and afterload variation on relative diastolic (A), systolic (B), and active (C) forces in *H. americanus*. Increasing the afterload significantly increased the diastolic force (p=0.0032, 2-way ANOVA), systolic force (p=0.0037, 2-way ANOVA), and the active force (p=0.013, 2-way ANOVA). At low afterloads, increasing the preload increased diastolic force ($p=9.2 \times 10^{-43}$, Spearman Rank), the systolic force ($p=8.8 \times 10^{-35}$, Spearman Rank), and active force ($p=8.8 \times 10^{-34}$, Spearman Rank). At high afterloads, the change in force due to preload was significant for diastolic force ($p=9.0 \times 10^{-39}$, Spearman Rank), systolic force (9.0×10^{-38}, Spearman Rank), and active force ($p=8.2 \times 10^{-26}$, Spearman Rank). Error bars represent one standard error.

Myosuppressin Experiments

The statistical significance of and between the treatment (myosuppressin or control), time (first or second trial), and afterload (high or low) were determined by three-way ANOVA, as seen in Table 2.

Table 2. The p values of the three-way ANOVAs for each parameter. Statistically significant values were labelled as follows: 0.01<p<0.05 with *, 0.001<p<0.01 with **, 0.0001<p<0.001 with ***, and p<0.0001 with ****. Treatment refers to comparison between myosuppressin and control, time refers comparison between first and second set of treatment, and afterload refers to the comparison between high and low afterload. P values greater than 0.1 were labeled not significant (ns).

Parameter	Treatment	Time	Afterload	Treatment x Time	Treatment x Afterload	Time x Afterload
Cardiac Output	****	***	0.0791	ns	***	0.0957
Frequency	****	ns	ns	ns	ns	ns
Diastolic Pressure	*	***	****	ns	0.05961	ns
Systolic Pressure	ns	****	****	0.0551	ns	**
Pulse Pressure	**	****	ns	0.0589	ns	ns
Diastolic Force	*	ns	ns	0.0754	ns	ns
Systolic Force	**	0.0503	ns	ns	ns	ns
Active Force	****	*	ns	ns	ns	ns

Cardiac output was significantly decreased due to increasing afterload ($p<0.0001$), treatment ($p<0.0001$), and time ($p=0.0004$, three-way ANOVA, Figure 14). Additionally, it was determined that there was an interaction between the variation treatment and afterload ($p=0.0006$, three-way ANOVA, Table 2).

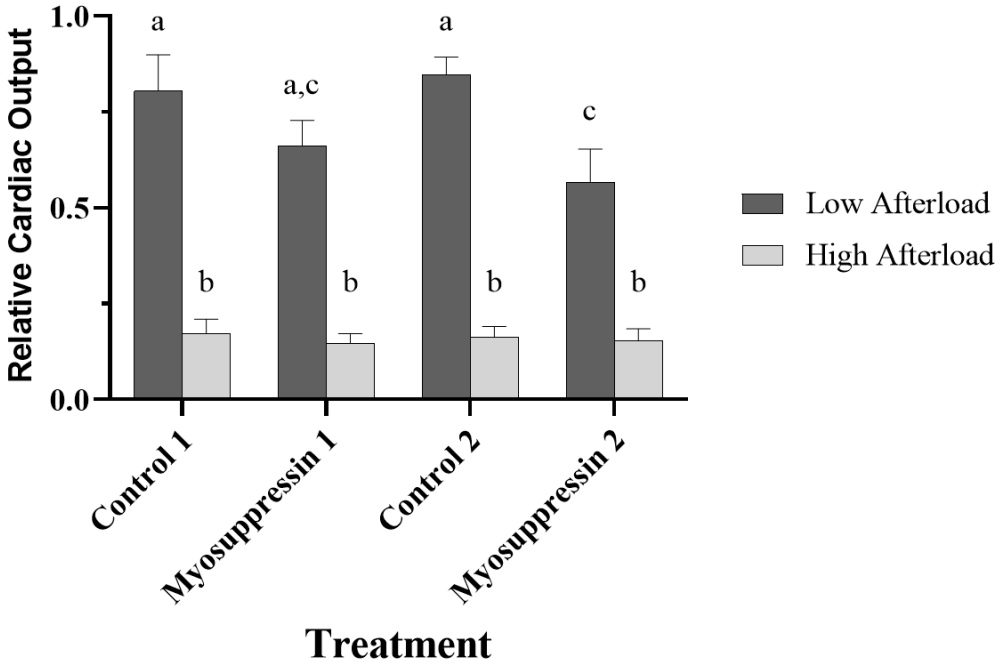

Figure 14. Cardiac output over two control and two myosuppressin trials at low and high afterloads. Post hoc analysis was included because of statistically significant interaction (Three-way ANOVA, treatment x afterload $p=0.0006$). Error bars represent one standard error, and different letters represent statistically significant values ($p<0.05$), and same letters represent statistically insignificant differences ($p>0.05$, post hoc analysis). Overall, cardiac output was significantly affected by treatment ($p<0.0001$), time ($p=0.0004$), and afterload ($p<0.0001$, Three-way ANOVA).

The frequency was significantly decreased by the addition of myosuppressin ($p<0.0001$, three-way ANOVA, Figure 15). Time and afterload did not have a consistent effect on the frequency, nor were there statistically significant interactions between the variables (Table 2).

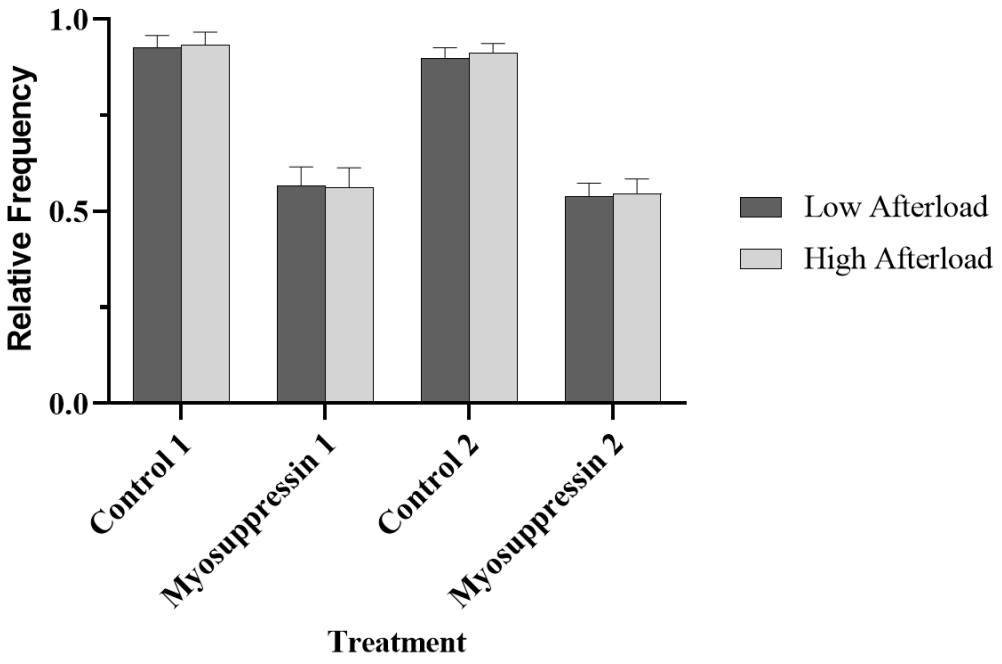

Figure 15. The frequency of the heart during two control and myosuppressin trials at high and low afterloads. The only significant source of variation for the frequency was due to the treatment ($p<0.0001$), whereas time ($p=0.1304$), and afterload ($p=0.7078$) did not overall effect frequency (three-way ANOVA). Error bars represent one standard error.

Time significantly impacted diastolic (Figure 16A, $p=0.0158$), systolic (Figure 16B, $p<0.0001$), and pulse pressure (Figure 16C, $p<0.0001$, three-way ANOVA), all pressures decrease over time. Treatment affected diastolic ($p=0.016$) and pulse pressures ($p=0.0076$), but not systolic pressures ($p=0.25$, three-way ANOVA). Afterload affected diastolic ($p<0.0001$) and systolic pressure ($p<0.0001$) but not pulse pressures ($p=0.53$, three-way ANOVA). Systolic pressure was significantly affected by an interaction between time and afterload ($p=0.0030$, three-way ANOVA).

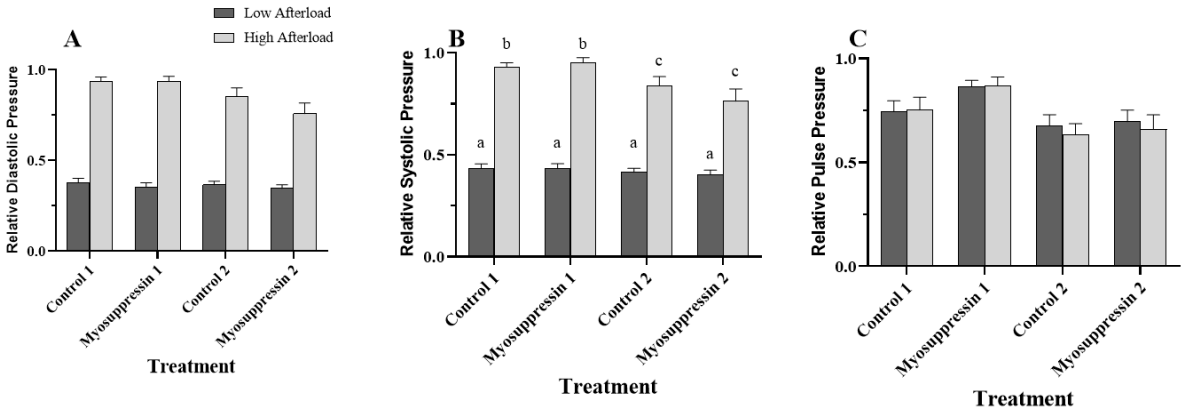

Figure 16. Diastolic (A), systolic (B), and pulse (C) pressures with and without myosuppressin at two afterloads. For diastolic pressure, treatment (p=0.0158), time (p=0.0003), and afterload (p<0.0001, three-way ANOVA) all had statistically significant effects. Afterload (p<0.0001), and time (p<0.0001), both significantly affected systolic pressure, but treatment (p=0.25 three-way ANOVA) did not affect systolic pressure, Treatment (p=0.0076) and time (p<0.0001) significantly affected pulse pressure, but afterload (p=0.53 three-way ANOVA) did not. Diastolic and pulse pressures had no statistically significant interactions (Table 2), but systolic pressure had a significant interaction between time and afterload (p=0.0030, three-way ANOVA). Error bars represent one standard error, and different letters represent statistically significant values (p<0.05), and same letters for different columns represent statistically insignificant differences (p>0.05, post hoc analysis).

Myosuppressin significantly increased the diastolic (Figure 17A, p=0.024), systolic (Figure 17B, p=0.0045), and active force (Figure 17C p<0.0001, three-way ANOVA) of the heart. Time and afterload had no statistically significant effect on diastolic (time: p=0.45, and afterload: p=0.79) or systolic forces (time: p=0.0503 and afterload p=0.33, three-way ANOVA). The active force was significantly affected by time (p=0.039) but not afterload (p=0.18, three-way ANOVA).

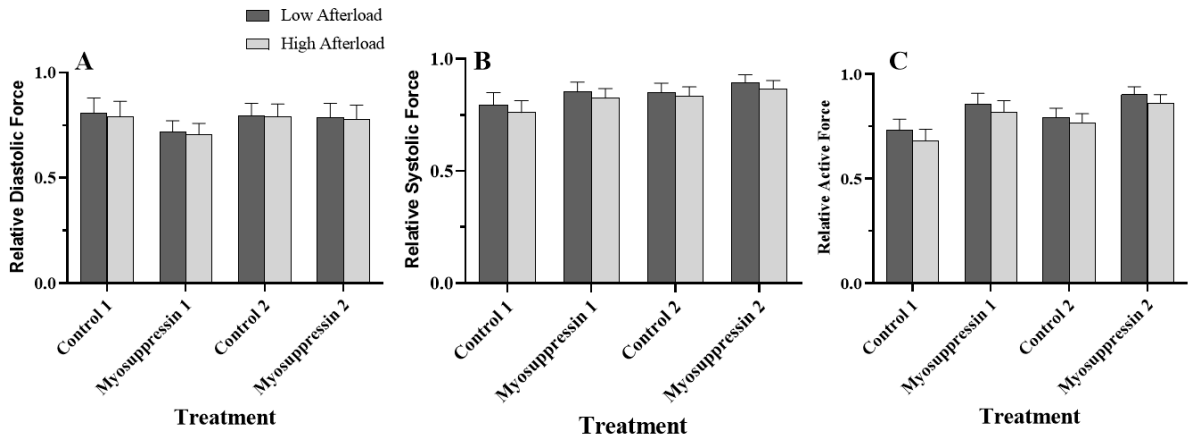

Figure 17. The relative diastolic, systolic, and active force over two control and two myosuppressin treatments. Treatment significantly affected diastolic (p=0.024), systolic (p=0.0045), and active forces (p<0.0001, three-way ANOVA). Time did not affect diastolic (p=0.45) or systolic force (p=0.0503), but did affect active force (p=0.039, three-way ANOVA). Afterload did not significantly affect diastolic (p=0.79), systolic (p=0.33), or active force (p=0.18, three-way ANOVA). There was no significant interaction of variables for diastolic, systolic, or active forces. Error bars represent one standard error.

Discussion

Evidence of a Baroreceptor-like Response

In mammals, the baroreceptor response maintains control over the cardiac output via regulation of the systemic resistance (afterload), and regulation of the heart's frequency and active force through neuromodulators and feedback loops (Figure 1). Thus, in a system with a baroreceptor-like response, we would expect to see a decrease in contraction force and frequency when afterload and pressure are increased. Our experiments on lobsters showed that the cardiac output decreased consistently across all lobsters when only the afterload was modulated (Figure 5). These decreases in cardiac output match those of Fickera (2019) in *H. americanus* and Wilkens and McMahon (1994) in *C. maenas*.

One of the key controls in the mammalian baroreceptor response is the heart rate. For example, dogs without baroreceptor nerves had a higher and more varied heart rate as compared to intact dogs (Cowley et al., 1973). In *H. americanus*, the heart rate change has been notoriously inconsistent in the Johnson/Ellers and Dickinson labs (Chin-Purcell, 2014; Dickinson, 2014; Fickera, 2019). For example, in Dickinson (2014), they found that in response to stretch the frequency could increase, decrease, or remain the same. We saw similar variation in our experiments. When only the afterload was manipulated, 50% of lobsters from the current experiment responded with a slight, but insignificant, increase in frequency, with an additional two outliers with significant increased frequency due to increased afterload. Fickera (2019) found the opposite, with 70% of lobster hearts decreasing, insignificantly, in frequency due to the increase in afterload.

In crabs, an increase in afterload by decreasing diameter of the tube intubating the sternal artery resulted in no changes in heart rate (Wilkens and McMahon, 1992). This result contrasts

with our current experiments, which has found that frequency can have a variety of responses to increased afterload. One potential reason for the divergence could be the sample size, because they completed this experiment on only five crabs. Over 18 lobsters, only three lobsters had a significant change from zero (Figure 6B), so five crabs may not have been a large enough sample size to encounter a crab that had a variable heart rate.

Similarly, Wilkens and McMahon (1994) found that increasing afterload had no significant effect on heart frequency, but that the semi-isolated nature of their dissection also caused there to be a 20-30% decrease in the frequency as compared to intact crabs. Increasing afterload of *H. americanus* typically did not cause frequency variation by more than 10% (with one lobster changing by 30%). The lack of change in heart rate in *C. maenas* could have been hidden by the overall heart rate change that it experiences due to being semi-isolated.

There was inter-lobster variation in heart rate response, which could be due to a variety of unknown or untracked factors, such as stage in molting cycle, or time since their last meal. For example, in humans the baroreflex was significantly reduced at high heart rates by temperature increases of about 1 °C (Crandall et al., 2000). Thus, even with a system as well understood as the human baroreflex, the system could be sensitive to a diverse set of variables.

Since increase in cardiac output was not correlated with changes in frequency, an alternative hypothesis is that increase in cardiac output could be a function of increased active force, which is consistent with our previously stated hypotheses. Similar to Fickera (2019), we found that the active force consistently decreased at higher afterloads (Figure 8C and 8F). However, there were large variations in the extent of our recorded decreases of active force and changes in frequency. For example, lobster 29 had the largest decrease in active force but also the largest increase in frequency. To examine the relationship more closely between active force

and frequency, we plotted the percent change go active force as a function of percent change of frequency (Figure 18). Although there is considerable variation, hearts that responded to increased afterload with larger decreases in active force tended to respond with a greater increase in contraction frequency. These results could indicate that the baroreceptor-like response is driven in lobsters by force modulation, with frequency acting as a fine tune modulation of the system.

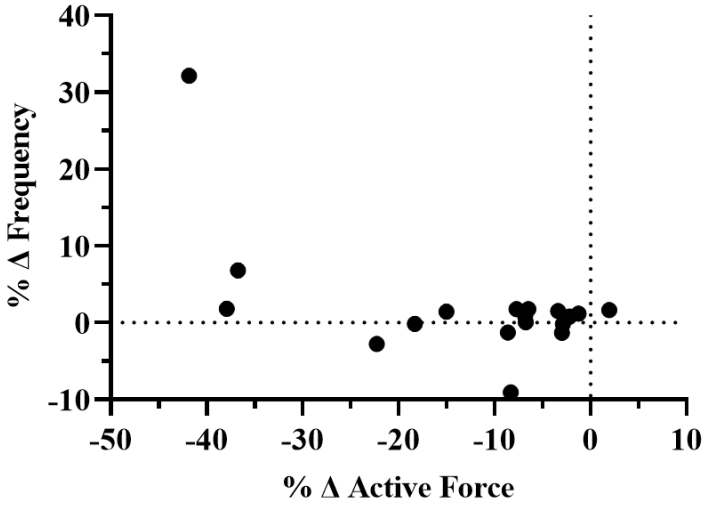

Figure 18. Examining the relationship between percent change of frequency and active force due to increase in afterload. More negative changes in active force tend to cause more positive responses in change in frequency.

Burggren et al. (1990) found that restrained *C. guanhumi* have baroreceptor-like responses, over short time frames with 5% increase or decrease in hemolymph levels. They determined that the crabs did not have a consistent heart rate response to the change in pressure levels. Their experiments were minimally invasive, which allowed the cardiovascular system to remain as intact as possible but limited the measurement of parameters. Our current experiments were much more invasive and produced less consistent results. Although more invasive, this dissection allowed there to be more control of the change in afterload and expanded the parameters that could be monitored, such as the cardiac output.

Effect of Preload on Frequency and Force

As preload increased, active force also increased, which shows that the muscle in the heart had a Frank-Starling Response (Figure 13C). The frequency of heartbeat has been seen to either increase, decrease, or not change in response to stretch, and all three possibilities are seen in experiments in which the lobster heart is stretched either bilaterally or uniaxially (Chin-Purcell, 2014; Dickinson, 2014), suggesting that the frequency response to stretch depends to some extent on the state of the lobster's system. These results were replicated in our current experimentation of uniaxially stretching the heart (Figure 10).

In the spiny lobster (*Panulirus japonicus*), preload was modulated in a multidirectional fashion by increasing the pressure of the saline entering the ostia (as opposed to pulling on arteries as in our experiments). Increasing the pressure in *P. japonicus* tended to cause active force and frequency to increase, although there was variability in the amount that each heart increased (Kuramoto and Ebara, 1984). In *H. americanus,* the frequency response could increase, decrease, or stay the same due to preload changes, but force increased significantly for diastolic, systolic, and active forces. The divergence between these results could indicate that these two species regulate heart preload through different mechanisms. Kuramoto and Ebara (1984) put forth an interesting theory for the inter-lobster variation, which is that even though they consistently modulated the pressure externally, there still was internal pressure variation, as an explanation for heart rate variation.

Other researchers have modulated preload by increasing the perfusion rate into crab hearts, which yielded similar results as increasing lobster heart preload. Six out of ten crabs had no heart rate change with increasing preload, and the other four had increased heart rate. Our experiments had a similar mix of results, heart rate did not decrease in any of the crabs in their

experiments, unlike in our current experiments (Wilkens and McMahon, 1992). Similar to the experiments in which only afterload was modulated, increasing the preload increased the active force, and the change in frequency was variable. In the afterload-only manipulation, both the active force and the cardiac output decreased with increasing afterload. With increasing preload, the active force increased, but cardiac output decreased.

At Low Afterloads, Increasing Preload Decreases Cardiac Output

Since the cardiac output is determined by stroke volume and frequency, one of those variables should explain the decrease in cardiac output at low afterloads (Figure 9). Since frequency did not change consistently with preload (Figure 11), changes in frequency did not explain the overall results seen in the cardiac output. Although stroke volume was not directly measured, we expect higher stroke volumes with higher active forces, but active forces increased with increasing preload, so higher active forces would not explain decreased cardiac output (Figure 13C). The pulse pressure was the only measured variable that decreases due to increased preload at low afterloads, or the only parameter to change with cardiac output (Figure 12C). Although pulse pressure also decreased at high afterloads, whereas cardiac output did not, this response caused by pulse pressure could have been hidden in the cardiac output because the whole system is at a higher pressure, so the pulse pressure variations were much smaller compared to the pressure experienced by the whole system. These results also suggested that even though the heart is pumping harder at higher preloads, the increased preload stretch prevented the saline from being pumped out of the heart. This effect could be due to the mechanics of the experiment, where stretch was imposed by pulling on arteries, which only allowed the heart to contract isovolumetrically.

Myosuppressin Increases Active Force and Decreases Frequency

Consistent with the result of previous work on isolated cardiac ganglion, whole heart, and stimulated heart muscle (Stevens et al., 2009), myosuppressin caused a decrease in frequency and an increase in active force on our heart-plus-arteries preparation. Unlike preload or afterload manipulation experiments, where frequency varied from lobster to lobster, myosuppressin caused heart rates to decrease significantly and consistently. Although both parameters changed significantly, the decrease in frequency was larger than the increase in active force (Figure 15 vs. Figure 17C). Interestingly, in Stevens et al. (2009), the frequency decreased by about 80% and the active force increased by about 50% in the whole heart preparation, whereas in our experiments, the frequency decreased by about 50% and the active force decreased by about 20%, even with similar concentrations of 10^{-7} M. This could be evidence for additional receptors in arteries that modulate the response of the heart. Additionally, the whole heart preparation of Stevens et al. (2009) did not pump against an afterload, which could have affected the change in active force.

In our preparation, we expanded on Stevens et al. (2009) preparation by exploring the effects of the change in frequency and active force on cardiac output, which is closely affected by both of those parameters. Even with the increased active force, the cardiac output decreased at high afterloads, which shows that the frequency is the main driver in the reduction of cardiac output in the myosuppressin experiments (Figure 14). At low afterloads, cardiac output was not affected by the addition of myosuppressin, which indicated that the increased active force is enough to compensate for the decreased frequency at low afterloads. At high afterload, the cardiac output is reduced, which implied that the frequency change in the baroreceptor-like response was mediated by neuromodulators.

The Effect of Time

The effect of time was most clearly seen in the myosuppressin experiments, where four sets of trials were recorded over about 2.5 h. In these experiments, pressure, cardiac output, and the active force all decreased over the course of the experiment (Table 2). Although we added glucose to the saline to stabilize hearts, it was only effective some of the time, thus, the data averages generally had a larger variability in the later times. Afterload-only experiments were conducted first in the lobsters used for afterload and preload experiments, so the variability due to exhaustion of the heart should be minimal. The preload data, which were almost always collected after the afterload-only data, may also exhibit more variation at highest preloads due to exhaustion of the heart, especially since these data were collected without glucose in the saline.

Conclusion

Our data support the hypothesis that there is baroreceptor-like control of the lobster heart, which is coarsely controlled by active force modulation. Heart rate could be used as a secondary control of cardiac output, if active force overcompensates, which creates a tightly regulated system. Increasing stretch caused a Frank-Starling Effect on the active force, but the cardiac output did not change proportionally, which indicates that the stroke volume is not only determined by the active force of the heartbeat, but also the amount that the heart is able to pump out at a given time. At high afterloads, myosuppressin essentially flipped the importance of the active force and frequency compared to the afterload-only experiments, with the frequency having a larger impact on the cardiac output compared to the active force. These data also demonstrated that there is neurological control of the baroreceptor-like reflex in the lobsters. Overall, *H. americanus* use both biomechanical and neurological control to maintain an optimal cardiac output under different physiological conditions.

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